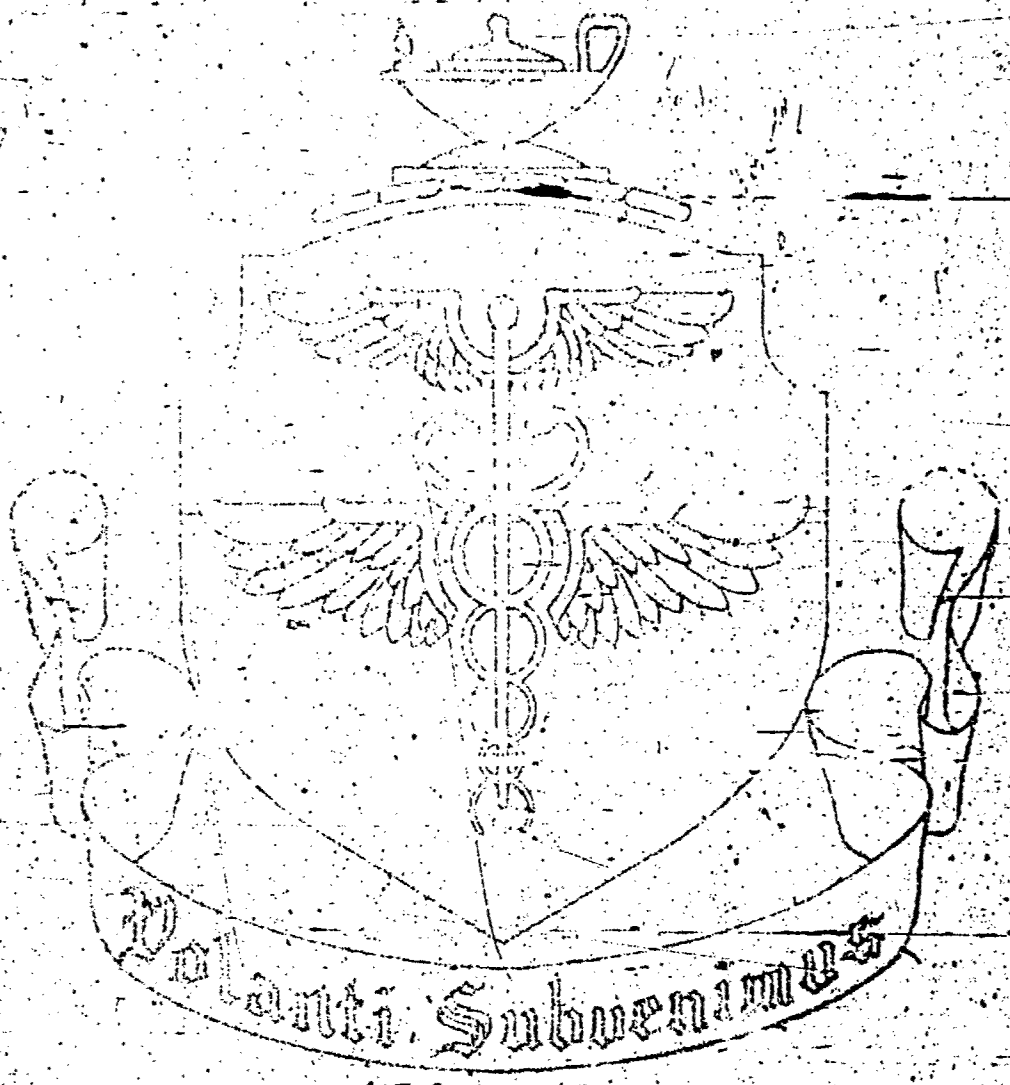

FOREWORD

This report was prepared at the Engineering Experiment Station, Georgia Institute of Technology, Atlanta, Georgia, by—

ROBERT H. FETNER, Ph.D.

ABSTRACT

Living neuroblasts of the grasshopper *Chortophaga viridifaciata* were exposed to ozone. The threshold dose necessary to produce a significant delay in mitosis was determined. Inhibition was reversible and mitosis, though delayed, went to completion. Cells dosed before early prophase were inhibited and differed from the controls in the time taken to reach anaphase. Cells in more advanced stages proceeded to anaphase at a time not significantly different from the controls.

Cells exposed in anaphase, telophase, interphase, and very early prophase were delayed in passing through midprophase and late prophase stages. Progression through preceding and succeeding stages was the same as control preparations. Exposure to ozone in the preprophase stages resulted in inhibition in late prophase, and cells exposed in later stages proceeded through the next cell division uninhibited. Stages sensitive to the threshold concentration of ozone are preprophase, and the inhibited stages are middle and late prophase.

This technical documentary report has been reviewed and is approved.

Robert B. Payne

ROBERT B. PAYNE
Colonel, USAF, MSC
Chief, Operations Division

MITOTIC INHIBITION INDUCED IN GRASSHOPPER NEUROBLASTS BY EXPOSURE TO OZONE

1. INTRODUCTION

Little information is available on specific cellular effects of ozone.

Investigations with microorganisms have been concerned mostly with the bactericidal, fungicidal, cysticidal, and virucidal properties of ozone. Giese and Christensen (14) in describing the cellular responses of protozoa, rotifer, and sea urchin eggs noted decreased movement of the organisms and swelling and blistering of the cytoplasm. They concluded that ozone penetrated the cell poorly and acted primarily as an oxidant against the plasma membrane.

In this laboratory, we have demonstrated the capacity of ozone to produce chromosome breakage in the root meristem cells of *Vicia faba* (12). Ozone is apparently selective against the nuclear material in the cell at concentrations below those which markedly affect the morphology of the tissues. The high percentage of chromosome aberrations produced by ozone and the type of dose-response correlation obtained indicate that this material is a very effective radiomimetic agent. Davis (11) has recently reported on the mutagenic activity of ozone in *Escherichia coli*, and Brinkman and Lamberts (3) have described the inactivation of oxidative enzymes in vivo after ozone inhalation in humans.

The investigations reported here are directed toward an expansion of our present knowledge of the cellular effects of ozone.

2. METHOD

Six-day *Chortophaga viridifaciata* embryos incubated at 37 C. (equivalent to 12-day embryos of Carlson's (4) at room temperature) were used in the experimental work. This material has several advantages: the work of previous investigators (4-10) has established the precision and noncyclic mitotic rate of these particular cells, and the large size and exceptional visibility make refined in vitro observations possible. Cultural procedures employed are essentially those developed by Carlson and his co-workers (4-10). As the thickness of such preparations precludes the effective use of phase contrast optics, all observations were made with ordinary microscopy. Details of cell division are clearly visible and a photographic sequence has been made of a single neuroblast cell through the entire mitotic cycle (13). The criteria used to determine the beginning of each mitotic stage are given in table 1.

To eliminate the temperature shock effect present in the experimental results of other investigators who used this material, it was decided to conduct all operations as close to the incubation temperature (38 C.) as would be feasible. To this end, a constant-temperature work space was constructed in which all the preparatory steps and dosimetry could be accomplished (fig. 1). Temperature control was maintained in inoculation box through thermostat control of the radiant-heating bottom surface. Heat is supplied through a 400-watt, radiant-heating wire located beneath a $\frac{1}{4}$-inch plate glass surface. This plate glass bottom acts as a continuous heat-radiating surface, and the high heat loss and continuous heat input of this system result in a small amplitude of temperature change while permitting the front of the box to remain open and considerable manipulation to take place through the open front. In operation, this box automatically

TABLE I

Criteria used to determine the various mitotic stages

Stage	Description of initiation of stage*	Duration* (min.)
Interphase	Chromatin granules are scattered in a homogeneous nuclear background. Nucleoli, very irregular, appear as grapelike clusters of small spheres.	27
Prophase		
Very early	Chromosome threads become visible among the nuclear granules.	24
Early	Nuclear granules disappear; the nucleus is filled with fine chromosome threads.	46
Middle	Threads increase in thickness and may be followed from one place in nucleus to another.	16
Late	About seven chromosomes are near the nuclear membrane in one-fourth optical cross section.	16
Metaphase	Nuclear membrane disappears; cell assumes a spherical shape.	13
Anaphase	Proximal ends of chromatids separate.	9
Telophase		
Middle	Cleavage furrow appears to be complete.	24
Late	Nucleoli lose their spherical shape; chromatin granules arranged in linear sequence.	33

*After Carlson et al. (10). The above description differs in the merger of certain stages, i.e., late plus very late prophase, prometaphase plus metaphase, and early plus midtelophase

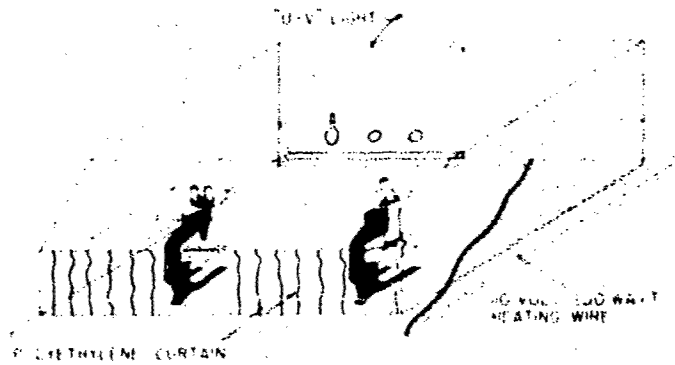

FIGURE 1

Inoculation box for temperature control.

maintains a temperature between 35° and 37° C., care being taken to keep the temperature from going above 38° C. By preparing samples under such conditions and then transferring them to the microscope incubator, the temperature shock effect was eliminated.

A new type of microscope incubator which offers several unique advantages was designed and put into operation. This incubator is an inflated polyvinyl bag through which hot air is forced in a closed circulating system. Forced-draft incubating systems offer good temperature control if the temperature differential is large enough between the desired incubator setting and room temperature. The flexible polyvinyl bag offers an additional advantage over rigid systems—namely, the microscope controls may be manipulated from the exterior of the bag without breaking into the closed forced-draft system. This is accomplished by pressing in on the bag and manipulating the controls directly through the polyvinyl material. This overcomes one of the more serious drawbacks to a forced-draft system—that of breaking into and disturbing the continuous air flow.

Ozone was generated (Welsbach Model T23) at room temperature from cylinder oxygen and scrubbed successively through an 0.8-normal sulfuric acid solution and then through a 2% potassium-iodide trap. Ozone to be used for dosimetry was that present as dissolved ozone in the 0.8-normal sulfuric acid solution. It has been shown in previous investigations that ozone is reasonably stable under such conditions—at least the rate of decomposition is minimized and is less than 10% over the period of the experiment. The concentration of ozone present in such a solution is a function of the distribution coefficient between the ozone and the gas immediately above it. This in turn is determined by the temperature. At, or close to, room temperature (23° C.) the distribution coefficient for ozone is about 0.24 (17). This means that at saturation the ozone concentration in solution will be about one quarter of that present in the gas which is being bubbled through it. The Welsbach ozonator is capable of producing ozone in concentrations from about 1 to 10% by weight from pure oxygen—depending on the voltage operation, rate of gas flow, and certain other variables. We have found that scrubbing ozone through 400 ml. of 0.8-normal sulfuric acid for 20 to 30 minutes produces a near-saturated solution and, as a matter of convenience, we allow this period of time for scrubbing. The final concentration of ozone is determined by varying the voltage on the ozonator or by diluting the resultant solution. After the ozone solution has been generated, it is poured into a glass-stoppered bottle. This particular bottle has a very narrow mouth, and the top has been ground smooth so that a tight fit will be obtained when a cover slip is inverted over the open neck. The bottle has a ground glass stopper, and special care is necessary to insure that no contaminating or reducing substances are present, as these would effectively reduce the ozone concentration. Several methods for ozone analysis have been evaluated in previous investigations (15). The ferrous-ferric system was selected as the best method for these experimental procedures. This analytic procedure involves the oxidation of the ferrous ion to the ferric ion by ozone:

$$2Fe^{++} + O_3 + 2H^+ \rightarrow 2Fe^{+++} + O_2 + H_2O$$

Enough ferrous ammonium sulfate in 0.8-normal sulfuric acid was added to the test solution to make a final concentration of approximately 0.5-normal ferrous ammonium sulfate solution. This is back-titrated with potassium permanganate standardized against sodium oxalate. Since this analysis is ordinarily conducted in dilute sulfuric acid solutions, it lends itself particularly well to our experimental procedure and eliminates the necessity of changing the pH during the analytical procedure as would be necessary, for example, with potassium iodide. Another advantage of this analytic method is the sharp end point evidenced in the permanganate titration.

In the dosimetry of the hanging-drop preparations, the embryos were exposed to ozone by inverting the preparations on the cover slip and placing them on the top of the sample bottle. Pains were taken to insure a complete seal between the cover slip and the ground lip of the bottle. Under these conditions, the concentration of ozone in the gas above the liquid

and in contact with the drop containing the embryo preparation may be estimated, based on the distribution coefficient of ozone at this temperature (17). After a specified contact time, the solution was replaced in the preparation, yolk material added, and observation begun immediately. This particular method of dosimetry seems to be dictated by several considerations: (1) the requirements of the embryo for a balanced physiologic solution, (2) the advantage of a short contact time, and (3) the necessity of eliminating reducing materials from the embryo preparations during ozone contact. It has been found in other investigations that ozone is not effective biologically until all reducing materials in solution have been eliminated. The presence of even small amounts of reducing agents provides marked protection from the ozone effect. Controls were run by exposing preparations in the same manner except that oxygen alone had been bubbled through the solution. The controls were *not* significantly different from Carlson's findings (10); table II shows the results of a typical control run.

TABLE II

Thirteen neuroblast cells treated with physiologic saline (control) in interphase

Mitotic stage	Average duration (min.)	Theoretic duration*
Prophase		
Very early	24 ± .4	24
Early	46 ± .9	46
Middle	16 ± .3	16
Late	16 ± .3	16
Metaphase	13 ± .3	13
Anaphase	9 ± .1	9
Telophase		
Early	24 ± .4	24
Late	33 ± .8	33
Interphase	27 ± .5	27

*After Carlson et al. (10).

Occasionally the embryo in a preparation would die, usually by drying up. This, however, did not confuse interpretation of the data, because, in such instances, the preparation became highly refractive and all the cells died. Any deviation from isotonicity is immediately detectable by changes in the optical characteristics of the preparations.

A 5-minute dose time was used in all the experiments to allow adequate time for diffusion of ozone into the cell and be short enough to permit accurate determination of the stage treated.

3. RESULTS

Threshold ozone concentration necessary to inhibit mitosis

A series of experiments was run to determine the concentration of ozone necessary to produce a significant inhibition of mitosis. The results are shown in table III.

Under these experimental conditions, no effect was detected until the embryos were exposed to an ozone atmosphere present in a closed system with a solution of 3.5 to 4.5 mg. per liter of ozone. In this concentration range there was a significant inhibition of mitosis; however, the effect was reversible and mitosis, although delayed, would go to completion. This concentration was used in all the dosimetry experiments. The lower concentrations of ozone undoubtedly represent the "ozone demand" of this particular solution. It was not feasible to work with higher concentrations of ozone because of the excessive duration of mitotic inhibition.

Mitotic stages sensitive to threshold concentration

Quantitative information on the effects of ozone on each of the specific stages of mitosis was obtained by replication. To this end, preparations were dosed and placed under the microscope, the preparations were mapped, and each cell was followed until the time of the next anaphase. The total time involved from

TABLE III

Duration of mitosis after exposure to various concentrations of ozone for 5 minutes

Ozone solution (mg./liter)	Number of cells	Average mitotic phase ($\bar{X} \pm \sigma(\bar{X})$) (min.)	Expected time (X = min.)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P*
0.0 to 1.5	18	208 ± 0.6	208	—	—
1.5 to 2.5	22	206 ± 0.8	208	0.25	.8
2.5 to 3.5	18	213 ± 4.0	208	1.2	.2
3.5 to 4.5	16	326 ± 6.0	208	19.0	< .01

*The probability values in this and the succeeding tables were determined by Student's t-test.

the end of dosing to the commencing of observation was 5 minutes; therefore, it is possible to predict the stage dosed, because all of the stages are at least twice this duration (table IV).

These data indicate that cells dosed earlier than early prophase (very early prophase, interphase, late telophase, midtelophase and early telophase) are inhibited by this dose of ozone and differ significantly from controls in the increment of time taken to reach the next anaphase. Cells more advanced at the time of ozone exposure than very early prophase proceed to anaphase at a time not significantly different from the controls.

Effect of ozone exposure on progression of mitosis

Individual cells were studied after ozone exposure, and their progress was followed from one mitotic stage to another. In each experiment from one to two cells were followed per preparation, the number to be followed depending on the number of cells in the particular

TABLE IV

Time to reach next anaphase of cells exposed to a solution of 3.5 to 4.5 mg. per liter ozone

Stage treated	Number of cells	Average time to next anaphase ($\bar{X} \pm \sigma(\bar{X})$)	Expected time ($\bar{X}$)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Interphase	9	204 ± 6	128	13	< .01
Very early prophase	8	115 ± 3	103	4	< .01
Early prophase	8	72 ± 2	68	2	.10
Midprophase	7	38 ± 1	37	1	.4
Late prophase	10	23 ± 1	23	0	—
Metaphase	5	5.4 ± 0.8	4.5	1.2	.4
Anaphase	6	313 ± 8	208	13	< .01
Midtelophase	6	225 ± 7	184	6.4	< .01
Late telophase	4	232 ± 7	158	10.3	< .01

stage to be studied that could be found in one microscope field. Therefore, the six cells used in each experimental group represent from three to six replicate experiments. The cells were exposed to the ozone for 5 minutes. The time was then recorded from the beginning of the next successive stage of mitosis and of each succeeding stage until the cells entered anaphase. Table V presents the data from those cells exposed in anaphase.

The duration of each succeeding stage, up to midprophase, was not significantly inhibited; however, once beyond this stage, the cells did proceed through metaphase to anaphase at the expected rate. Table VI presents the results when cells were treated in midtelophase. The cells progressed through successive stages at the expected rate until midprophase and late prophase where there was a significant inhibition. Then they proceeded through metaphase to anaphase as in the controls.

Cells treated in late telophase (table VII) proceeded as the controls to midprophase where there was a significant inhibition in midprophase and late prophase, and then proceeded at the expected rate to anaphase.

TABLE V

Six cells treated in anaphase

Mitotic stage	Average duration ($\bar{X} \pm \sigma(\bar{X})$)	Expected duration (X)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Midtelophase	26 ± 1	24	2.0	.10
Late telophase	32 ± 1	33	1.0	.4
Interphase	26 ± 1	27	1.0	.4
Prophase				
Very early	24 ± 0.4	24	0	—
Early	46 ± 1.0	46	0	—
Middle	32 ± 1.0	16	16	< .01
Late	48 ± 1.0	16	32	< .01
Metaphase	13 ± 0.4	13	0	—

TABLE VI

Six cells treated in midtelophase

Mitotic stage	Average duration ($\bar{X} \pm \sigma(\bar{X})$)	Expected duration (X)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Late telophase	35 ± 0.6	33	3.3	.02
Interphase	25 ± 0.6	27	3.3	.02
Prophase				
Very early	25 ± 0.4	24	2.5	.05
Early	45 ± 1.0	46	1.0	.4
Middle	26 ± 0.5	16	20	< .01
Late	49 ± 1.1	16	32	< .01
Metaphase	13 ± 0.3	13	0	—

TABLE VII

Six cells treated in late telophase

Mitotic stage	Average duration ($\bar{X} \pm \sigma(\bar{X})$)	Expected duration (X)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Interphase	25 ± 0	27	2.5	.05
Prophase				
Very early	26 ± 0.8	24	2.5	.05
Early	50 ± 1.5	46	3.3	.02
Middle	31 ± 0.9	16	17	< .01
Late	51 ± 1.2	16	27	< .01
Metaphase	13 ± 0.5	13	—	—

TABLE VIII

Six cells treated in interphase

Mitotic stage	Average duration ($\bar{X} \pm \sigma(\bar{X})$)	Expected duration (X)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Prophase				
Very early	23 ± 1.0	24	1.0	.40
Early	49 ± 1.4	46	2.1	.10
Middle	21 ± .3	16	16.6	< .01
Late	51 ± 1.0	16	35.0	< .01
Metaphase	14 ± 0.8	13	1.2	.25

TABLE IX

Six cells treated in very early prophase

Mitotic stage	Average duration ($\bar{X} \pm \sigma(\bar{X})$)	Expected duration (X)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Prophase				
Early	43 ± 1.4	46	2.1	.10
Middle	22 ± 0.7	16	8.6	< .01
Late	29 ± 0.5	16	26.0	< .01
Metaphase	13 ± 0.3	13	13	

Cells treated in interphase (table VIII) also proceeded at the expected rate to midprophase and late prophase where again there was a significant inhibition.

Cells treated in very early prophase (table IX) were inhibited in midprophase and late prophase and then proceeded at the expected rate to anaphase.

Cells treated in early prophase (table X) proceeded to anaphase without inhibition as did cells treated in middle and late prophase (tables XI and XII).

4. DISCUSSION

Mitosis is a physiologic process. The progression of a cell through division is a function of physiochemical processes acting within genetically determined parameters. Each of the various mitotic stages is dependent for its predetermined completion on events in previous stages as well as events transpiring in the specific stage. Mitosis provides an exquisitely sensitive as well as a fundamentally significant test process.

Quantitation in such studies is rendered difficult by the limited number of biologic systems

TABLE X

Six cells treated in early prophase

Mitotic stage	Average duration ($\bar{X} \pm \sigma(\bar{X})$)	Expected duration (X)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Prophase				
Middle	18 ± 1.0	16	2.0	.10
Late	19 ± 1.5	16	2.0	.10
Metaphase	14 ± 0.5	13	2.0	10

TABLE XI

Six cells treated in midprophase

Mitotic stage	Average duration ($\bar{X} \pm \sigma(\bar{X})$)	Expected duration (X)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Late prophase	15 ± 0.6	16	1.6	.20
Metaphase	13 ± 0.4	13	—	—

TABLE XII

Six cells treated in late prophase

Mitotic stage	Average duration ($\bar{X} \pm \sigma(\bar{X})$)	Expected duration (X)	Significance ratio $\frac{\bar{X} - X}{\sigma(\bar{X})}$	P
Metaphase	12 ± 0.8	13	1.2	.30

of adequate visibility where noncyclic mitosis occurs. Marine and amphibian eggs have been studied extensively, although intracellular visibility is poor; mammalian cell suspensions in the exponential growth phase is one recently developed system; and the neuroblast mitosis used in this study is another.

The inherent difficulty of studying a dynamic system with static technics has imposed additional limitations on such investigations, and continuous observation of the living cell appears to be a necessary requirement. Evidence of mitotic stage reversal after irradiation (6) demonstrates the fallacy of assumptions based on data obtained from slide preparations.

In these investigations, it was found that ozone produced a specific effect on mitosis. At the threshold concentration cells exposed in anaphase, telophase, interphase, and very early prophase were delayed significantly in passing through the next middle and late prophase stages. Cells more advanced than very early prophase proceeded to anaphase at a time not significantly different from the controls. It thus appears that the stages sensitive to ozone under these conditions are preprophase, and the inhibited stages are middle and late prophase. These data suggest that the mitotic activity of ozone is similar in some respects to the responses to high-energy irradiation, but there are significant differences. Exposure to ozone does not result in a reversion of prophase stages which has been described for irradiation (6). With low irradiation doses, reversion is limited to cells in late prophase, and there is an accumulation of midprophases. With higher doses, all prophase cells revert to an interphase condition. Thus, with irradiation, we have a critical stage, late prophase, and a reversion process which is dose-dependent. The higher the dose, the greater is the reversion. This reversion phenomenon was absent with ozone exposure; however, late prophase was the stage in which the mitotic inhibition was expressed.

Ozone is of particular interest in irradiation biology because the decomposition of ozone in solution produces the same active radicals formed in water by high-energy irradiation. The chemical activity of free radicals produced by the catalytic decomposition of hydrogen peroxide with ferric ions has recently been discussed (16, 19) and Phillips (18) describes the production of chromosome aberrations in barley by these hydrogen peroxide precursers. From studies of the kinetics and mechanisms of ozone decomposition, Akler and Hill (1) assumed that in solution the reactions

$$HO_3^- + OH \rightarrow 2HO_2 \qquad (1)$$

$$O_3 + HO_2 \rightarrow OH + 2O_2 \qquad (2)$$

are responsible for disappearance of ozone, are relatively slow, and determine the rate of reaction. Also, these authors indicate that reaction represents an equilibrium which is maintained so long as ozone is present (1). The decomposition of ozone in solution is catalyzed by hydroxyl ions and is dependent on temperature. The evidence presented in this study suggests that only a part of the irradiation effect on mitosis can be explained in terms of active radical formation in water. Such products produced externally to the cell do not cause a reversion of the prophase stages of mitosis.

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Unclassified Report

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